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TITLE: Barriers to Therapy: A Novel 3-D Model to Study the Effect of Tumor Interstitial Pressure on Endocrine-resistant Breast Cancer

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14. ABSTRACT
The breast cancer microenvironment poses a formidable barrier to effective therapy. The pathophysiologic mechanisms underlying treatment resistance in breast cancer are not known; in part this is due to the lack of a suitable, well-characterized model. Our purpose was to develop a 3D breast cancer culture system capable of reproducing the tumor microenviroment. We cultured MCF-7, a human breast cancer cell line, under three conditions: on polystyrene, on a two-dimensional collagen gel, and on a 3-dimensional collagen gel. The extent of phenotypic abnormality was assessed by changes in morphology on phase-contrast images and alterations in gene expression by qRT-PCR. Expression of E-cadherin, c-kit, MMP-2, MMP-12 and MMP-19 in MCF-7 grown on polystyrene was significantly higher than in MCF-7 grown in the three-dimensional collagen gel. TGF-beta expression was significantly higher in MCF-7 cells grown in the three-dimensional collagen gel compared to polystyrene. Gene expression in the two-dimensional gels trended towards that seen in the 3D gels but was not statistically significant. MCF-7 cells grown in 3D formed small, loose aggregates in comparison to the stellate appearance of cells grown on polystyrene. We can non-destructively grow and image live breast cancer cells. We were able to build an improved, preciselydefined synthetic scaffold that better replicates the gene expression changes seen in human breast cancer, demonstrate that three-dimensional growth affects the expression of genes important in cell-cell adhesion and cell invasion, and non-destructively image these cells.

15. SUBJECT TERMS
Breast Cancer, in vitro culture, three dimensional, metastasis.

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INTRODUCTION:

High tumor interstitial pressure (TIP) in solid neoplasms poses a formidable barrier to effective therapy[1]. The pathophysiologic mechanisms underlying elevated TIP in breast cancer are not known; in part this is due to the lack of a suitable, well-characterized *in vitro* model. To date, few *in vitro* breast cancer models have systematically replicated TIP, effectively investigated how TIP affects the influx of therapeutic agents, or conclusively demonstrated how TIP changes with alterations in cytokine signaling, adhesion and mechanical stress. Relying on recent advances in the field of tissue engineering, we can construct sophisticated organotypic three-dimensional (3D) cell culture systems to examine the tumor microenvironment changes during response to chemotherapy, including tumor shrinkage and regression[2]. These changes in the extracellular matrix influence cell growth and viability via integrin-mediated transmembrane mechanotransduction, but this has not been tested in a systematic fashion in a dynamic 3D breast cancer tumor model.

We hypothesize that high tumor interstitial pressure acts via an NFkappaB pathway to block macromolecular diffusion of therapeutic agents into breast neoplasms, allowing for cell growth and the appearance of treatment resistance. We developed a 3D culture system capable of exposing cultured breast cancer cells to a range of mechanical microenvironments. We are able to non-destructively measure the TIP *in situ* and image the live cells using optical methods.

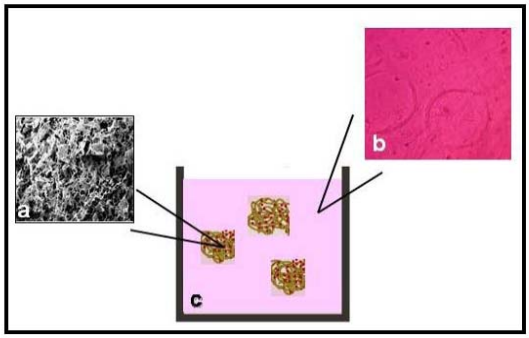

Figure 1: Micrographs of (a) the stiffer microporous collagen mesh inclusions seeded with epithelial tumor cells and (b) the softer nanoscale collagen gel seeded with mesenchymal cells; (c) is a schematic of the tumor-like inclusions suspended in 3D.

Using the BCRP funding, we built an improved, precisely defined synthetic scaffold to more closely replicate the mechanical microenvironment of breast cancer tumors. Specifically, we have successfully co-cultured human breast cancer cells in stiff collagen inclusions suspended in fibroblast seeded compliant type I collagen gels. We are now in the process of applying for follow on funding to use this system to replicate tumor interstitial pressure, demonstrate that high TIP inhibits macromolecular diffusion, and prove that this is mediated by NFkappaB activation as suggested by the work of others[3, 4]. We anticipate that this will ultimately lead to better understanding and targeted treatment in patients with treatment-resistant breast cancer.

BODY: Accomplishments, progress and lack of progress are described for each experiment in the statement of work below.

Experiment 1: *Determine the effect of high TIP on cell growth in our novel 3D breast cancer model.*

We have developed a novel 3D culture model consisting of tumor-like inclusions (type I collagen meshes seeded with breast cancer cells) suspended in type I collagen gel seeded with human umbilical vein endothelial cells and human dermal fibroblasts (**Figures 1 and 2**). Protocols are in **Appendix A**. In order to verify that breast cancer cells behave differently on the molecular level in a three-dimensional culture environment, we carried out a series of gene expression studies using MCF-7 cells.

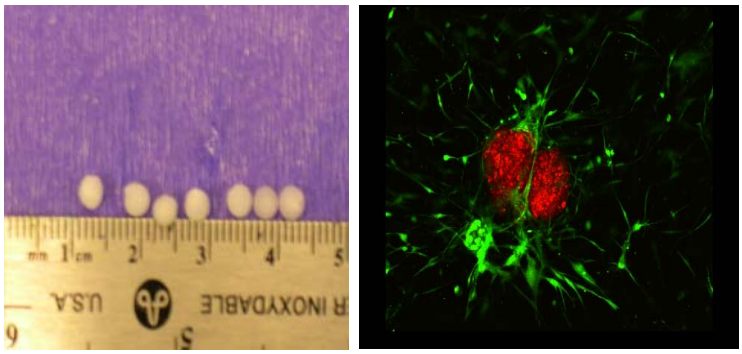

Figure 2: Left, collagen type I mesh inclusions formed by the phase separation method. Right, confocal image of breast cancer cell seeded collagen inclusions (red) surrounded by a fibroblast seeded gel (green).

We cultured MCF-7, a human breast cancer cell line, under three conditions: on polystyrene, on a two-dimensional collagen Type 1 gel, and on a 3-dimensional collagen Type 1 gel. The extent of phenotypic abnormality was assessed by changes in morphology on phase-contrast images and alterations in gene expression by qRT-PCR. G3PDH was used as an endogenous control, and relative quantification was performed using MCF-7 cells grown on polystyrene alone as the calibrator or reference sample. Genes tested included E-cadherin, c-kit, TGF-beta, MMP-2, MMP-12 and MMP-19, chosen for their importance in cell-matrix signaling.

Expression of E-cadherin, c-kit, MMP-2, MMP-12 and MMP-19 in MCF-7 grown on polystyrene was significantly higher than their expression in MCF-7 grown in the three-dimensional collagen Type 1 gel. TGF-beta expression was significantly higher in MCF-7 cells grown in the three-dimensional collagen Type 1 gel compared to polystyrene. Gene expression in the two-dimensional gels trended towards that seen in the three-dimensional gels but was not statistically significant. MCF-7 cells grown in 3D formed small, loose aggregates in comparison to the stellate appearance of cells grown on polystyrene. The complete dataset is included in the charts in **Appendix B**.

As an intermediate step toward the study of TIP, we used the 3D culture system to look at gene expression changes in cells as a function of hypoxic conditions. For these pilot studies, we used human thyroid cancer cells in addition to the MCF7 cells, since these cells are used routinely in the Rosen Laboratory. The thyroid data are summarized here in the text, and the complete data summary is included in **Appendix C.**

Although its role in thyroid tumorigenesis is still unknown, hypoxia inducible factor 1 (HIF-1) has been reported as an important predictor of tumor progression for various solid cancers. Traditionally, gene expression analysis on markers of hypoxia, including HIF-1 and carbonic anhydrase IX (CA-9), has been done on polystyrene (PS) cell cultures. Yet, PS cultures fail to reproduce the three-dimensional nature of human tumors. This study examines the expression of

HIF-1 and CA-9 in hypoxic, three-dimensional cultures of human follicular and papillary thyroid carcinomas. Two human thyroid neoplasm cell lines (WRO and KAT5) were cultured under hypoxic microenvironments in three different conditions: on PS alone, on a 2D collagen type 1 gel and in a 3D collagen type one gel. Cell lines were cultured for 48 hours under at least 2% hypoxia. Rapid Trizol extraction of total RNA was performed followed by conversion to cDNA. qRT-PCR for HIF-1 and CA-9 was performed using G3PDH as the endogenous control. Relative quantification was completed and gene expression calculated as fold difference compared to PS normoxia controls.

In hypoxic PS cultures, expression of HIF-1 significantly decreased by about 50% and expression of CA-9 significantly increased by at least 15 times as compared to normoxia controls, both at 24 and 48h. The expression of HIF-1 was significantly lower and CA-9 higher in cultures grown in 3D compared to PS. Gene-expression in 2D trended towards that seen in PS but was not statistically significant, suggesting that collagen itself does not alter gene expression. HIF-1 expression in human thyroid cancer cell lines appears to inversely correlate with chronic hypoxia; furthermore, under hypoxic conditions, three-dimensional growth appears to abrogate rising CA-9 expression. Ultimately, we hope that these gene expression changes seen in our 3D model will provide a platform for better understanding treatment resistant thyroid cancer.

Experiment 2: *Evaluate the relationship between TIP and macromolecular diffusion using our 3D model.*

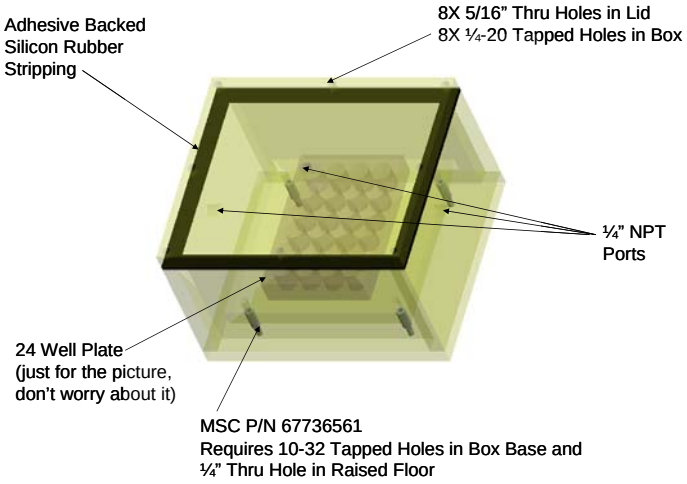

Figure 3: Schematic drawing of the pressure box designed and constructed for the variable pressure experiments.

We have not been successful making TIP measurements on the inclusions. The instrumentation required is not available off the shelf, and needs to be designed and built from scratch[5], an endeavor requiring much more time and money than available in this exploratory grant. In response to this setback, we built and constructed pressure boxes to apply varying hydrostatic pressures to the entire culture systems using calibrated mass flow controllers (**Figure 3**). We are currently using the pressure boxes to perform experiments that will relate the applied external pressure to the amount of macromolecular diffusion using FITC labeled dextran. We are applying a measured amount of FITC-labeled dextran to the culture and measuring the fluorescence recovery after photobleaching as an estimate of macromolecular diffusion across our tumor inclusions. This work is proceeding slowly, as the technical demands of the imaging procedure far exceeded our expectations.

Experiment 3: *Characterize the role of NFkB activation in TIP and cell growth.*

We have not completed any work under this experiment due to our initial failure at measuring TIP inside of the type I collagen inclusions. We will be able to look at NFkB activation using the pressure box system described above, but we do not expect this data to directly answer the question about this particular pathway activation. In order to get direct evidence of a relationship between TIP and NFkB activation, the wick method of TIP measurement needs to be developed. We are currently seeking follow on funding to carry out this work, since the required instrumentation will be much more expensive than initially anticipated.

KEY RESEARCH ACCOMPLISHMENTS:

- Three dimensional cell culture system was developed, demonstrated, and characterized.
- Non-destructive imaging was carried out on live cells in the new culture system.
- Incubation chambers were designed and built to vary the external pressure on the three-dimensional tissue analogs during cell growth.
- Gene expression studies using human breast cancer and thyroid cancer cell lines were carried out under temperature stress and hypoxic conditions.
- Gene expression studies using human breast cancer and thyroid cancer cell lines are currently underway in high and low hydrostatic pressure conditions.
- Persons receiving pay from this research effort:
    - Dr. Cassandra Noack, Ph.D. Postdoctoral Fellow, Klapperich Laboratory (12 months)
    - Dr. Susana Wishnia, M.D. Surgical Resident, Rosen Laboratory (10.5 months)
    - Dr. Nirmal Bhogal Ph.D. Postdoctoral Fellow, Rosen Laboratory (1.5 months)

REPORTABLE OUTCOMES:

1. Abstracts

Susana C. Wishnia, MD, Sandeep Patel, Cassandra L. Noack, PhD, Catherine Klapperich, PhD, and Jennifer E. Rosen, MD. ATA Paper #3778 *78th Annual Meeting of the American Thyroid Association* (October 4 - 7, 2007). "Expression of hypoxia-inducible factor 1a and carbonic anhydrase IX in human thyroid cancer"

2. Presentations

Sandeep Patel, Susana C. Wishnia, MD, Cassandra L. Noack, PhD, Nirmal Bhogal, PhD, Catherine Klapperich, PhD, and Jennifer E. Rosen, MD. "The effect of three-dimensional growth on epithelial-to-mesenchymal transition in human breast cancer cells," Poster Presentation #148 at the *60th Annual Meeting of the Society of Surgical Oncology*, Washington, DC, March 2007.

3. Employment applied for and received based on training supported by this award.

Dr. Cassandra Noack applied for and received a job at Alkermes, Inc. in Cambridge, MA as a result of her postdoctoral training in the Klapperich and Rosen Laboratories. She began employment in April 2007.

CONCLUSIONS:

We have designed, fabricated and field tested a novel three-dimensional cell culture system for the study of breast cancer. We have used this system to look a MCF7 human breast cancer cells in experiments under heat stress and hypoxic conditions. We extended the work to include several thyroid cancer cell lines. Three postdoctoral researchers were trained under this grant.

HIF-1α expression in human thyroid cancer cell lines appears to inversely correlate with chronic hypoxia. CA-9 expression increases with prolonged hypoxia on PS; however, in both cell lines three-dimensional growth appears to abrogate rising CA-9 expression under hypoxic conditions. Expression of E-cadherin, c-kit, MMP-2, MMP-12 and MMP-19 in MCF-7 grown on polystyrene was significantly higher than their expression in MCF-7 grown in the three-dimensional collagen Type 1 gel. TGF-beta expression was significantly higher in MCF-7 cells grown in the three-dimensional collagen Type 1 gel compared to polystyrene. Gene expression in the two-dimensional gels trended towards that seen in the three-dimensional gels but was not statistically significant. MCF-7 cells grown in 3D formed small, loose aggregates in comparison to the stellate appearance of cells grown on polystyrene.

We can non-destructively grow and image live breast cancer cells using optical methods. We were able to build an improved, precisely defined synthetic scaffold that better replicates the gene expression changes seen in human breast cancer, demonstrate that three-dimensional growth affects the expression of genes important in cell-cell adhesion and cell invasion, and non-destructively image these cells.

We were unable, during the course of the funding period, to measure TIP inside of the collagen type I inclusions. We are continuing efforts to measure the effect of hydrostatic pressure on the simulated tumors using pressure control boxes. We are also pursuing follow on funding to build the system necessary to measure TIP inside of the inclusions. The instrumentation was more expensive and involved than we initially anticipated.

Overall, we feel that this has been a successful year, and that the funding provided has allowed us to start a collaboration we would not have otherwise been able to commence. Ultimately, we hope that the altered gene expression changes seen in our 3D model will provide a platform for better understanding of treatment resistant breast cancer.

REFERENCES:

1. Stohrer, M., et al., *Oncotic pressure in solid tumors is elevated.* Cancer Res, 2000. **60**(15): p. 4251-5.
2. Nelson, C.M. and M.J. Bissell, *Modeling dynamic reciprocity: Engineering three-dimensional culture models of breast architecture, function, an neoplastic transformation.* Seminars in Cancer Biology, 2005. **15**: p. 342-352.
3. Zahir, N., et al., *Autocrine laminin-5 ligates alpha6beta4 integrin and activates RAC and NFkappaB to mediate anchorage-independent survival of mammary tumors.* J Cell Biol, 2003. **163**(6): p. 1397-407.
4. Zhou, Y., et al., *The NFkappaB pathway and endocrine-resistant breast cancer.* Endocr Relat Cancer, 2005. **12 Suppl 1**: p. S37-46.
5. Ozerdem, U. and A.R. Hargens, *A simple method for measuring interstitial fluid pressure in cancer tissues.* Microvasc Res, 2005. **70**(1-2): p. 116-20.

APPENDIX A: Protocols Developed for this Study

A.1 Collagen Sphere Protocol

1. In lyophilizer jars, add a 50ml centrifuge tube with about 20ml liquid N_2.
2. Add a bench cover or paper towel within the jar to add padding under the tube.
3. Using a 2-20ul pipettor, add 15ul of sonicated Col-GAG solution into an ice bucket of liquid N_2 from a height of approximately 15cm above the liquid level (slightly above the N_2 vapour height at which the pipette tip will freeze). The spheres will float for about 10sec before sinking to the bottom of the bucket. Once the appropriate number of spheres has been formed, pour off most of the liquid N_2 and then allow the remainder to boil off. Keep a close eye on the spheres during this process since they must remain frozen.
4. Once the liquid N_2 has boiled off, pour the spheres into the centrifuge tubes within the lyophilizer jars and lyophilize at 300 torr for 48hr.

A.2 Making Collagen Gel Solution (10 mL) Cellular and Acellular (1 - 2 hours)

The 2D and PS experiments will be plated on a 6 well plate and the 3D on a 24 well plate

NB: - Keep collagen gel in freezer/fridge until needed
- Must have stocks of 1M NaOH, 1M HCl, 10X PBS
- Amount of collagen gel solution made is dependent upon the plate used, i.e. 24 wells – 1 mL/well; 12 wells – 2 mL/well; 6 wells – 3 mL/well
- If not making 10 mL, then take the number of mL you are making and divide by 10, then multiply all numbers in recipe by this quotient (i.e. making 8 mL of collagen, multiply all numbers by 0.8)
- Acellular and cellular collagens must be made slightly differently, see "a and b" protocols below

- Ingredients for 10 mL collagen solution:
  - 6 ml collagen
  - 250 uL 10X PBS
  - If making **3D**→3.25 mL media + 0.5 mL of media with appropriate amount of resuspended cells or if making **2D**→3.75 mL total media
  - pH paper (neutralize to approximately 7.4, use NaOH→try not to overshoot)

1. Label 50 mL Falcon tube

2. Pipette 250 uL of 10X PBS into a 50 mL Falcon tube

3. Add 3.25 mL of media to this 50 mL Falcon tube

4. Add 6 mL of collagen (from fridge)

5. Neutralize pH to 7.4
   - Slowly add 10 uL of NaOH, watch solution (solution = DMEM + phenol red; phenol red buffers at 7.5, i.e. acidic = yellow, basic = magenta pink), keep adding base till solution is very light pink; periodically check pH with pH paper (between 7.0 to 7.4 is optimal)
   - Pipette 5 uL from solution onto the pH paper→ Do Not put paper into the tube

6. Once solution is at a pH of 7.4, set aside

If making: 3D gels follow "a" protocol
2D gels follow "b" protocol

"a" Protocol for 3D Gels

7a. Resuspending cells in 0.5 mL of solution

- This is a tricky procedure in terms of calculations. One must be careful to take into account the final concentration of cells in the collagen solution. The concentration of cells in the 0.5 mL of solution does not matter. If you want to pipette 1mL of collagen solution into a 24-well plate and each well is supposed to have a cell density of approximately 1e6 cells, then you must do the following for a 10 mL collagen solution:
  - to have a concentration of 1e6 cells/mL, you need to mix 10e6 cells with 10 mL of collagen solution.
  - in order to get 10e6 cells, you must go back to the solution you finished making in step 8 above in the cell counting procedure and pipette out 10 mL of this solution into a new 50 mL Falcon tube (10 mL of this solution contains 10e6 cells because the concentration is 1e6 cells/mL)
  - centrifuge the new Falcon tube (1,200 rpm for 10 minutes) and vacuum aspirate the supernatant, keep the pellet (pellet = 10e6 cells)
  - add 0.5 mL of media to this pellet and resuspend

8a. Add the 0.5 mL of resuspended cells in media to the pH 7.4 solution and check pH again

- you have just put 10e6 cells into 10 mL of collagen (i.e. 1e6 cells/ mL of collagen solution)

9a. Make sure to put the collagen back into the fridge

10a. Aliquot freshly made 2D/3D collagen solution with cells into wells (do not use micropipetter, use the electric pipetter)

11a. Check freshly laid gels under microscope for normality

12a. Place into incubator for 20 minutes, to allow collagen gel to harden

13a. Add about 1 mL of media to the top of each of the collagen gels and place in incubator under appropriate conditions (39 C, 1% Oxygen, Increased Pressure, microgravity, etc.) and for appropriate time

14a. Change media every 24 hours

- **NB**: DO NOT VACUUM ASPIRATE the media, gently tilt and micropipette the media off the 3D gels, and then add 1 mL of the appropriate media, and incubate again.
- **If you are using media for hypoxia experiment, vacuum suction O2 out of media prior to use**.

"b" Protocol for 2D Gels

7b. Add 0.5 mL of media to the pH 7.4 solution from step 6 above, and check pH again

8b. Make sure to put the collagen back into the fridge

9b. Pipette (using micropipetter) drops of acellular collagen solution onto the bottom of each well→do this very sparingly, because you are trying to create the thinnest layer of collagen humanly possible, you may not even be able to see the layer.

10b. Shake the entire plate and add drops of collagen to fill gaps in the collagen layer

11b. Incubate for approximately 20 – 60 minutes to allow gel to harden

12b. Pipette appropriate concentration and volume of cells onto the thin collagen layer. (for example a 6 well plate needs 3 mL of cell suspension per well; therefore, if you need 250,000 cells per well, you need to create a cell suspension solution of 250,000 cells/3 mL or approximately 83,000-84,000 cells/mL, and then pipette 3 mL of this solution on the thin collagen layer evenly)

13b. Check freshly laid gels and cells for normality

14b. Incubate in appropriate conditions (39 C, 2% Oxygen, Increased Pressure, microgravity, etc.) and for appropriate time

15b. Change media every 24 hours

- **NB**: DO NOT VACUUM ASPIRATE the media, gently tilt and micropipette the media off the 3D gels, and then add 1 mL of the appropriate media, and incubate again

APPENDIX B: MCF-7 3D Platform Validation Study Data Summary

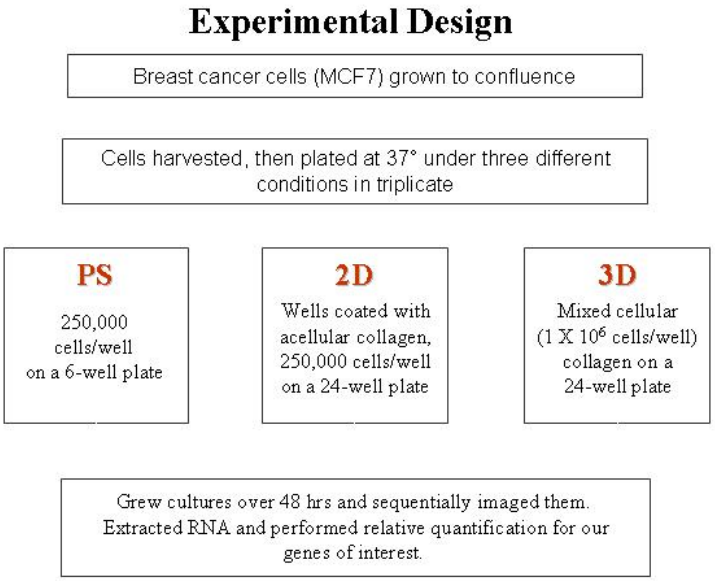

Figure B.1 Schematic of the experimental design.

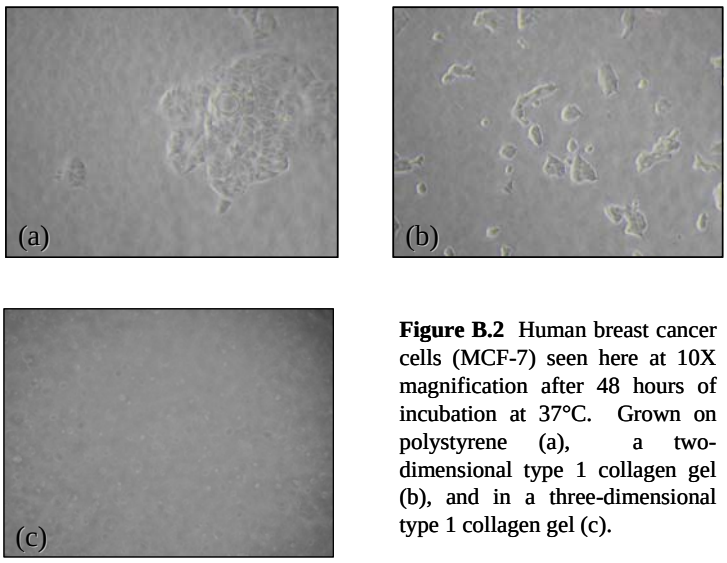

Figure B.2 Human breast cancer cells (MCF-7) seen here at 10X magnification after 48 hours of incubation at 37°C. Grown on polystyrene (a), a two-dimensional type 1 collagen gel (b), and in a three-dimensional type 1 collagen gel (c).

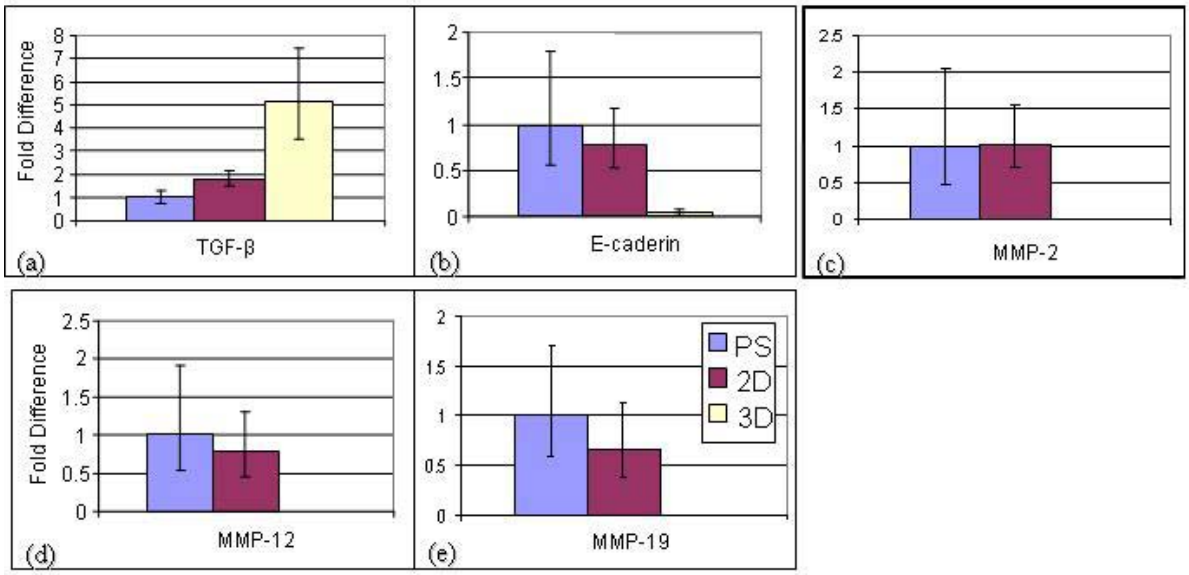

Figure B.3. The expression of TGF-β (a), E-cadherin (b), MMP-2 (c), MMP-12 (d), and MMP-19 (e) in human breast cancer cells (MCF-7) grown in a three dimensional collagen type 1 gel (3D) versus polystyrene (PS). Graph (a) demonstrates an increase in TGF-β gene expression in cells grown in 3D versus on PS, and graphs (b) – (d) show decreased gene expression in cells grown in 3D versus those grown on PS. Data is from qRT-PCR expression relative to the cells grown on PS.

APPENDIX C: Hypoxia Study Data Summary

C.1 Methods

Cell growth: Seven human thyroid neoplasm cell lines (WRO, NPA, KAT-5, KAT 10, KAK-1, ARO and TPC) were maintained in the following conditions: for the ARO cell line, DMEM medium in 5% fetal calf serum, 1X glutamine, 1X penicillin/streptomycin (P/S); for NPA, WRO, and TPC cell lines, in RPMI 1640 medium in 10% fetal calf serum, 1X glutamine, 1X P/S; for KAT-5, KAT-10, and KAK-1 cell lines, RPMI without phenol red, 10% fetal calf serum, 1X P/S, 1X glutamine, and 5ml 1X MEM non-essential amino acids 10nM. All seven cell lines were incubated at 37 degrees in a humidified atmosphere of 5% CO2. They were cultured in three different conditions: on polystyrene alone, on a two-dimensional collagen type 1 gel and in a three-dimensional collagen type 1 gel for 48 hours, during which time they were sequentially imaged to assess changes in morphology.

Measurement of cadherin production: After 48 hours, TRIzol® Reagent extraction of total RNA will be performed with the assistance of the Qiagen Mini kit (Valencia, CA) followed by conversion to cDNA using Superscript III (Invitrogen, Carlsbad, CA). Real-time PCR was performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) for E-cadherin, c-kit, AP2-alpha, MMP2, MMP12, and thyroglobulin; G3PDH will serve as the endogenous control. The data was analyzed using the Applied Biosystems 7000 System SDS software. Primers and probes for the genes E-cadherin, thyroglobulin and for G3PDH was designed with the Primer Express software (version 2.0; Applied Biosystems, Foster City, CA) and the gene assays for c-kit, AP2-alpha, MMP2, and MMP12 from Applied Biosystems Taqman Gene Assays (Foster City, CA). Normal human thyroid RNA (Ambion, Austin, TX) was used as the calibrator sample for relative quantification and gene expression was calculated as fold difference compared to the expression in normal human thyroid. Each cDNA sample was tested in triplicate, and the mean values calculated. The delta-delta Ct relative quantification technique was used to compare gene expression levels.

C.2 Results

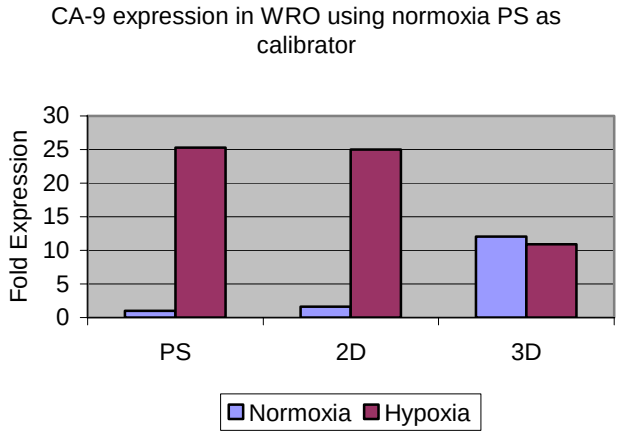

Figure C.1: CA-9 qRT-PCR results for WRO human thyroid cancer cells in the 3D culture system.

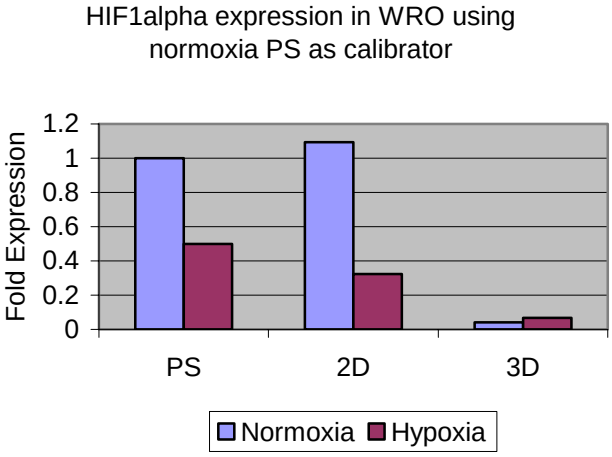

Figure C.2: HIF1alpha qRT-PCR results for WRO human thyroid cancer cells in the 3D culture system.

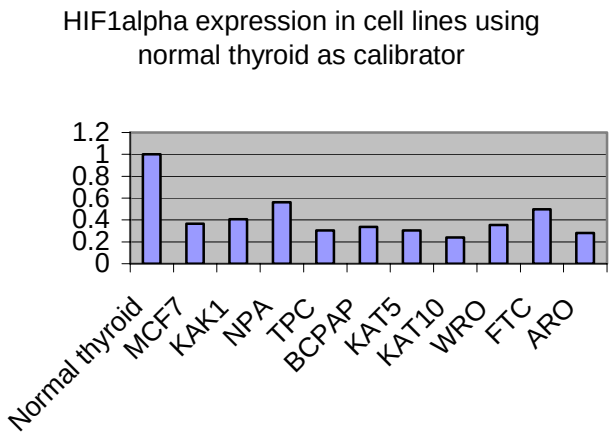

Figure C.3: HIF1alpha qRT-PCR results for MCF7 and human thyroid cancer cells in the 3D culture system.

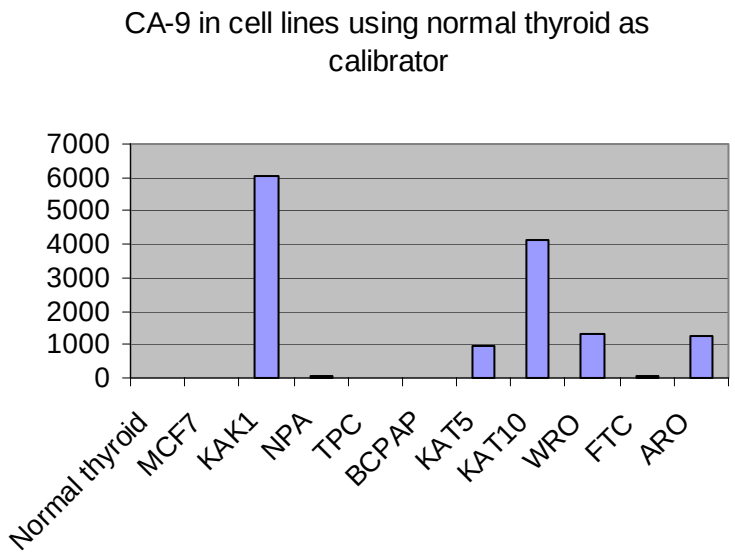

Figure C.4: CA-9 qRT-PCR results for MCF7 and human thyroid cancer cells in the 3D culture system.